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Synthesis, characterization, anti-cancer and anti-bacterial study of isatin conjugated 4-azidopyrazoline derivatives

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ABSTRACT

*New derivatives of isatin conjugated 4-azidopyrazolines were synthesized **5(a-e)** and characterized by physical and spectral data. From p-aminoacetophenone, 1-(4-azidophenyl)ethanone **(1)** was prepared by diazotization and subsequent reaction with sodium azide. Claisen-Schmidt condensation of compound **(1)** with different aromatic/hetero aldehydes gave chalcones **2(a-e)** which were converted into pyrazolines **3(a-e)** using hydrazine hydrate and treated with chloroacetyl chloride to give 1-(3-(4-azidophenyl-5-(4-aryl/hetero aryl)-4,5-dihydro-1H-pyrazol-1-yl)-2-chloroethanones **4(a-e).** Condensation of compounds **4(a-e)** with isatin gave the final compounds **5(a-e).** The synthesized compounds were evaluated for anti-bacterial and in vitro anti-cancer properties. Among the five compounds, **5b&5d** showed significant activity against p.aeruginosa with MIC values at 6.25 and 12.5 µg/ml. In MTT assy for cytotoxicity activity, compound **5a** showed highest activity against NCI-H23 and MCF-7 cell lines with IC_{50} values at 1.06 & 3.92 µM/ml, while the other compounds were moderately active with IC_{50} values >20 µM/ml. In vivo anticancer activity studies against Ehrlich ascites carcinoma (EAC) revealed that compound **5a** has highest increase in life span (% ILS) and mean survival time (MST) with 73.47 % and 31.5 ± 0.48 days respectively. A good correlation was observed between the results of in vitro and in vivo anticancer study of the compounds.*

Key words: Isatin, 4-azidopyrazolines**,** Hybridization approach, Anti-bacterial activity, Cytotoxic activity, *In vivo* anticancer activity.

INTRODUCTION

Pyrazoles/pyrazolines are an interesting class of heterocyclic compounds with synthetic versatility and significant biological activities such as analgesic & anti-inflammatory **(1-4)**, anti-diabetic **(5)**, anti-microbial **(6-9)**, anti-tubercular **(10-11)**, anti-viral **(12-13)**, anti-proliferative **(14-15)**, anti-convulsant **(16)** etc. In recent years, a number of reports have been documented and few patents have been filed on pyrazole and pyrazoline derivatives as potent anticancer agents particularly for B-Raf kinase **(17-21)** and EGFR kinase inhibitor activities **(22-23)**. On the other hand, Isatin is a core moiety in many of the marketed anti-cancer drugs and as well as in dyes, pesticides and analytical reagents. Literature survey reveals that various derivatives of isatin have diverse biological activities like antibacterial **(24)**, antifungal **(25)**, antiviral **(26)**, anti-mycobacterial **(27)**, anticancer **(28-29)** and anticonvulsant **(30)**. In the design of new drugs hybridization and bioisosterism are the key approaches that would help in obtaining new molecules with improved biological activity corresponding to lead compounds. Thus, by adopting hybridization approach, a new series of isatin conjugated pyrazoline derivatives were synthesized bearing azido (N_3) group on phenyl ring in all the compounds. The structures of newly synthesized compounds were confirmed on the basis of physical and spectral data. Compounds were screened for anti-bacterial activity using cup-plate method and anti-proliferative activity was carried out using MTT assay method. All compounds were tested for *in vivo* anticancer activity against Ehrlich acsites carcinoma (EAC) induced mice.

MATERIALS AND METHODS

All the solvents and chemicals used were of synthetic grade from SD fine chemicals, E.Merck, NR chemicals and Aldrich chemicals. Completion of the reactions was monitored by analytical thin layer chromatography (TLC) using E- Merck 0.25 mm silica gel plates. Visualization was accomplished with UV light (256 nm) and iodine chamber. Purification of synthesized compounds was done by re-crystallization process. The purity of the compounds was checked by a single spot in TLC. Melting points were determined in open capillary tubes using ANALAB melting point apparatus and are uncorrected. All the ^{1}H NMR spectra were recorded on AVANCE 300 MHz spectrometer using DMSO-d_6 as solvent and tetra methyl saline (TMS) as an internal standard. Chemical shift values are listed in δ scale. The ^{13}C NMR spectra of synthesized compounds were recorded on Varian Gemini 100 MHz spectrophotometer. The IR spectra were recorded on Shimadzu FTIR spectrophotometer by using 1% potassium bromide discs. Mass spectra of the compounds were recorded on Agilent 6430 mass spectrophotometer.

General procedure for synthesis of 1-(4-azidophenyl)-3-(aryl/ heteroaryl) prop-2-en-1-ones 2(a-e)
Equimolar quantities of 1-(4-azidophenyl) ethanone (1) and aryl/heteroaryl aldehyde were taken in 90% aq. ethanol and to which added, a required quantity of 10% aq. KOH solution and stirred for 12-16 hr at room temperature in order to obtain a precipitate. The reaction was monitored by TLC and the precipitate obtained was filtered, washed thoroughly with water and recrystallized from 90% aq. ethanol.

General procedure for synthesis of 3-(4-azidophenyl)-5- (aryl/heteroaryl)-4, 5-dihydro pyrazoles 3(a-e)
A mixture containing compound **2** (0.01 mol) and 99% hydrazine hydrate (0.02 mol) was taken in 15-20 ml of aq. ethanol and heated to reflux for 10-12 hr. The completion of the reaction was monitored by TLC and the reaction mixture was poured into ice-cold water. The precipitate obtained was filtered, washed thoroughly with water and recrystallised from 90% aq. ethanol.

General procedure of 1-(3-(4-azidophenyl-5-(aryl/heteroaryl)-4, 5-dihydro-1H-pyrazol-1-yl)-2-chloroethanones 4(a-e)
To a mixture containing compound **3** (0.01 mol) and triethyl amine (0.03 mol) in 20 mL of dry benzene, was added chloroacetyl chloride (0.015 mol) drop by drop while stirring in ice-bath and then bringing the reaction mixture to room temperature. Stirring was continued for the next 2-3 hr. The benzene was removed by rota evaporator in order to give a solid residue which was washed with sodium carbonate solution to decompose excess chloroacetyl chloride. The crude product obtained was washed thoroughly with water and without recrystallization, proceeded for the next step.

General procedure for synthesis of 1-(2-(3-(4-azidophenyl)-5-(4-aryl/heteroaryl)-4, 5-dihydro-1H-pyrazol-1-yl)-2-oxoethyl) indoline-2,3-diones 5(a-e)
A mixture containing compound **4** (0.01 mol), isatin (0.01 mol) and anhyd. K_2CO_3 (0.02 mol) was taken in 15-20 mL of DMF and stirred at 60^0c on a magnetic stirrer for 4-5 hr. The reaction mixture was dumped into ice-cold water and extracted with CH_2Cl_2. The solvent was evaporated and the crude residue obtained was recrystallized from methanol.

PHARMACOLOGICAL SCREENING
IN VITRO **CYTOTOXIC ACTIVITY**
The cytotoxic activity of synthesized compounds was evaluated against three cancer cell lines NCI-H23, MCF-7 and HCT-116 as described in the literature **(31-32)**. Cancer cell lines were grown in DMEM media supplemented with 20 % heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, 100 mg/mL streptomycin and 2 mM-Glutamine. The cell cultures were maintained in a humidified atmosphere with 5 % CO_2 at 37 °C. The cells were subcultured twice a week, seeding at a density of about 2×10^3 cells/mL. Cell viability was determined by the trypan blue dye exclusion method. 5×10^3 cells/well were inoculated in 96-well microtiter plate for 24 h, before treatment with the compounds, cells were allowed to attachment with wall of the plate. Test compounds were dissolved in dimethyl sulfoxide (DMSO), further diluted with saline to appropriate volume. Different concentrations of the compound under test (6.25, 12.5, 25, 50 and 100 μM/mL) were added to the wells, and the cells were incubated at 37 °C in a 5 % CO_2 incubator for 48 hr. The cells were treated with 10 μL MTT dye solution (5 mg/mL) for 4 h cultivation. The media along with the MTT solution was washed with 100 μL of DMSO solution. The absorbance of formazan solution was measured at 540 nm with an automatic multi-well plate reader (Victor3TM, Perkin-Elmer, and USA). Percentage inhibition of proliferation was calculated as a fraction of control (without drug).

%Growth = Absorbance of test/Absorbance of control×100.
% Inhibition = 100 - % Growth.

IN VIVO **ANTITUMOR ACTIVITY**

Male Albino Swiss mice weighing between 20-25 g were selected for screening of anti-cancer activity. Animals were kept and maintained under standard laboratory conditions and were provided free access to food and water ad libitum. Animals were allowed to acclimatize to the laboratory environment for seven days prior to *in vivo* experiment. The selected animals were divided into eight groups, each group comprising of twelve animals. EAC cells were procured from the donor mice and were suspended in a known quantity of 0.9 % NaCl (Normal saline). The cell count was adjusted to 2×10^6 cells/mL. All the groups were treated with EAC cells through intraperitonial (ip) route except the normal control group on day zero. Group I was treated with normal saline (5 mL/ kg body weight) and group II as EAC control. Group III was treated with standard drug 5-Fluorouracil (20 mg/kg body weight). The test compounds were administered through ip at dose of 50 mg/kg body weight in groups IV- VIII, respectively. All the test compounds and 5-Fluorouracil were treated daily for nine days starting from first day after tumor transplantation. After nine days, six animals from each group were sacrificed. The tumor volume and cell count parameters were recorded. Mean survival time (MST) for remaining six mice of each group was recorded **(33)**. The groups and design of experiment:

Group I: Normal saline
Group II: EAC (2×10^6 cells)
Group III: EAC (2×10^6 cells) + 5-Fluorouracil
Group IV: EAC (2×10^6 cells) + Compound **5a**
Group V: EAC (2×10^6 cells) + Compound **5b**
Group VI: EAC (2×10^6 cells) + Compound **5c**
Group VII: EAC (2×10^6 cells) + Compound **5d**
Group VIII: EAC (2×10^6 cells) + Compound **5e**

TUMOR WEIGHT CELL COUNT

The five mice were dissected and the total ascetic fluid was harvested from peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube and the packed cell volume noted by centrifuge at 1000 rpm for 5 min and viable cells were checked by trypan blule dye exclusion test and cells were counted in Neubauer counting chamber.

MST AND PERCENTAGE INCREASE IN LIFE SPAN (%ILS)

The activity of the pyrazoline compounds **5(a-e)** on tumor growth was evaluated by recording the mortality within the observation period. Percentage increase in life span (ILS) was calculated by using the following formula.

% ILS = (MST of treated group - MST of control group ×100) / MST of control group
MST = (Survival time of each mice in a group (days)) / Total number of mice

ANTI-BACTERIAL ACTIVITY

The Anti-bacterial activity of synthesized compounds was assessed by determining the MIC **(34)**, which is defined as the lowest concentration of the compound that completely inhibited the growth of each strain after overnight incubation. In the present study, MIC was determined by placing graded doses of compounds in petri plates containing solidified sterile agar medium which was inoculated with the test organisms. After suitable incubation, inhibition zones were observed according to the concentration. 10 mg of each test compound was taken in vial. Then 10mL of DMSO was added. From the stock solution a series of concentrations were prepared. The test bacteria grown at 37^0C in nutrient agar medium was diluted in sterile nutrient broth medium in such a manner that the suspension contain about 10^7 cells/mL. This suspension was used as the inoculum. Nutrient agar medium was prepared and transferred into boiling tubes each containing 20ml and sterilized by using autoclaving. Sufficient level of inoculum was transferred into medium when it is at 45°c and plating was done. After solidification of the medium cavities were made in each plate by using sterile stainless steel borer. The cavities were filled with different concentrations of test compounds. After allowing all plates for diffusion kept for incubation at 37°c for 24 hrs. After incubation inhibition zones were observed and MIC was determined. The above process was performed for the solvent (blank) and the standard drug. A similar experiment with medium, DMSO and inoculum without compound was also performed to ensure that the DMSO has no inhibitory effect in the dilutions used.

RESULTS AND DISCUSSION

The different steps involved in the synthesis were given under **Scheme 1**. The starting compound 1-(4-azidophenyl) ethanone **(1)** was prepared from p-aminoacetophenone by diazotization and subsequent treatment of diazonium salt with sodium azide. Further, compound **(1)** was converted into chalcones by Claisen-Schmidt condensation with different aromatic/hetero aromatic aldehydes in presence of aq. KOH. IR spectra of the compounds **2(a-e)** showed

absorption bands around 1650 cm^{-1} due to α, β-unsaturated carbonyl group. Cyclisation of compounds **2(a-e)** with hydrazine hydrate gave corresponding pyrazolines **3(a-e)** in good yields and structures were confirmed by FTIR, mass and ^{1}HNMR. In IR spectra, the shifting of the absorption peaks from 1650 to around 1600 cm^{-1} indicated the ring closure and the formation of pyrazoline ring. Moreover, a typical absorption around 3300 cm^{-1} due to N-H stretching of pyrazoline ring was also observed. In addition, the mass spectra recorded for these compounds clearly supported the structures which correspond to their molecular weights. ^{1}HNMR spectra of compounds **3(a-e)** showed a prominent AMX pattern for three pyrazoline protons by appearing as three double doublets around δ 3.2, 3.6 & 5.4 due to CH_2 and CH protons of pyrazoline ring and NH proton of pyrazoline appeared as singlet around δ 10.0. Treatment of compounds **3(a-e)** with chloroacetyl chloride reagent in presence of triethylamine base gave corresponding1-(3-(4-azidophenyl-5-(4-aryl/hetero aryl)-4, 5-dihydro-1*H*- pyrazol-1-yl)-2-chloroethanones **4(a-e).** The absorption bands around 1660 cm^{-1} due to carbonyl stretch of amide and disappearance of N-H absorption band clearly indicated the formation of compounds. Further, the reaction of compounds **4(a-e)** with isatin in DMF afforded the title compounds **5(a-e)** that were confirmed by spectral data. Presence of three carbonyl absorptions around 1730 cm^{-1} (C=O of isatin), 1670 (C=O of isatin) and 1660 (C=O of amide) in FTIR and three double doublets (AMX system) due to CH and CH_2 protons of pyrazoline ring and a singlet around δ 5.0 due to two protons of CH_2CO in ^{1}HNMR spectra clearly indicated the formation of compounds. The aromatic protons were observed at the expected chemical shifts between δ 6.7- 8.0. ^{13}C NMR spectra of compounds showed a peak around δ 184.0 due to carbonyl group (C-23) of isatin, the carbon attached to pyrazoline nitrogen (N-C=O) appeared around δ 170.0 and the other carbon of carbonyl group of isatin (C-22) appeared at δ 162.0. The aromatic carbons appeared in the range of δ 117.0-160.0. The C-3, C-4 and C-5 of pyrazoline ring appeared around δ 150.0, 40.0 and 59.0 respectively. Moreover, all the compounds exhibited molecular ion peaks of 100 % intensity in EI-MS spectra corresponding to their molecular weights which further confirmed the structures.

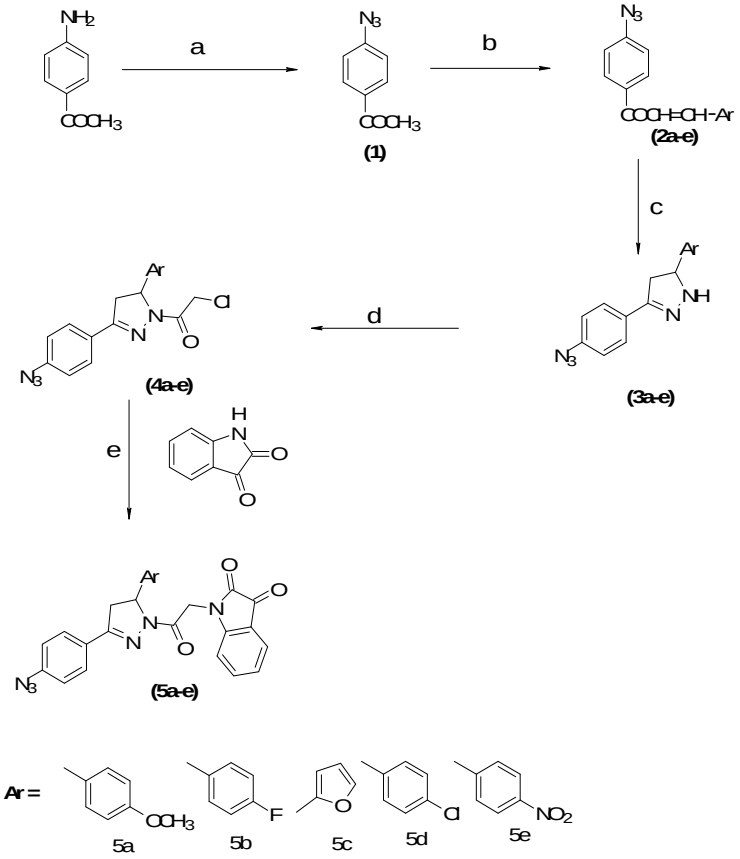

Figure 1: The total synthetic path way. Reagents and conditions: (a) $NaNO_2$, conc HCl, NaN_3; (b) ArCHO, aq. Ethanol; (c) NH_2NH_2. H_2O, aq. Ethanol; (d) TEA, dry benzene, $ClOCCH_2Cl$; and (e) DMF, anhyd K_2CO_3
Scheme-1

Physical and spectral data of synthesized compounds

1-(2-(3-(4-azidophenyl)-5-(4-methoxy phenyl)-4, 5-dihydro-1H-pyrazol-1-yl)-2-oxoethyl) indoline-2, 3-dione (5a)

Yellow solid; yield: 74%; mp: 119-121^{0}c; IR (KBr, cm^1): 1736 (C=O of isatin), 1679 (C=O of isatin), 1664 (C=O of amide), 1616 (C=N) and 1085 (C-O-C); ^{1}HNMR (DMSO-d_6, 400 MHz, δ ppm): 6.8-7.8 (12H, Ar-H), 5.5-5.6 (dd, 1H, pyrazoline), 4.9-5.1 (s, 2H, CH_2CO), 3.8-3.9 (dd, 1H, pyrazoline), 3.8 (s, 3H, OCH_3) 3.2 (dd, 1H, pyrazoline); ^{13}C NMR (DMSO-d_6, 100MHz, δ ppm): 185.6 (C=O of isatin, C-23), 172.0 (C=O of N-C=O, C-6), 161.4 (C=O of

isatin, C-22), 157.6 (C-O-CH_3, C-17), 151.O (C-3 of pyrazoline), 147.0 (C-N_3, C-11), 134.4, 130.8, 129.6, 128.8, 124.8, 122.3, 117.6, 114.2, 59.0 (C-5 of pyrazoline), 55.8 (O-CH_3, C-34), 54.0 (CH_2, C-20), 40.0 (CH_2 of pyrazoline, C-4); MS (m/z): 480.47(M-H); Anal.Calcd. for $C_{26}H_{20}N_4$Calculated C: 64.99, H: 4.20, N: 17.49. Found C: 64.89, H: 4.10, N: 17.39.

1-(2-(3-(4-azidophenyl)-5-(4-flurophenyl)-4,5-dihydro-1H-pyrazol-1-yl)-2-oxoethyl)indoline-2,3-dione (5b)
Yellow solid; yield: 85%; mp: 121-125^{0}C; IR (KBr, cm^{-1}): 1738 (C=O of isatin), 1674 (C=O of isatin), 1666 (C=O of amide), 1601 (C=N) and 1086 (C-F) cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz, δ ppm): 6.7-8.0 (12H, Ar-H), 5.4-5.6 (1H, dd, pyrazoline), 4.9-5.1 (s, 2H, CH_2CO), 3.8-3.9 (dd, 1H, pyrazoline), 3.2 (1H, dd, pyrazoline); ^{13}C NMR (DMSO-d_6, 100MHz, δ ppm): 185.8 (C=O of isatin, C-23), 171.0 (C=O of N-C=O, C-6), 160.4 (C=O of isatin, C-22), 160.0 (C-F, C-17), 151.2 (C-3 of pyrazoline), 146.8 (C-N_3, C-11), 134.2,130.6, 129.4, 128.6,124.7, 122.4, 117.6, 114.2, 58.9 (C-5 of pyrazoline), 54.0 (CH_2, C-20), 40.1 (CH_2 of pyrazoline, C-4); MS (m/z): 468.43(M-H); Anal.calcd. for $C_{25}H_{17}N_6O_3F$ Calculated C: 66.81, H: 3.81, N: 18.70. Found C: 66.78, H: 3.72, N: 18.65.

1-(2-(3-(4-azidophenyl)-5-(2-furan-2-yl)-4,5-dihydro-1H-pyrazol-1-yl)-2-oxoethyl)indoline-2,3-dione (5c)
Brown solid; yield: 75%; mp: 124-129^{0}C; IR (KBr, cm^{-1}): 1738(C=O of isatin), 1669 (C=O of isatin), and 1620 (C=O of amide); ^{1}HNMR (DMSO-d_6, 400 MHz, δ ppm): 7.1-7.9 (12H, Ar-H), 5.2-5.4 (dd, 1H, pyrazoline), 4.9-5.0 (s, 2H, CH_2CO), 3.5-3.6 (dd, 1H, pyrazoline), 3.0 (dd, 1H, pyrazoline); ^{13}C NMR (DMSO-d_6, 100MHz, δ ppm): 184.4 (C=O of isatin, C-28), 171.2 (C=O of N-C=O, C-17), 160.3 (C=O of isatin, C-27), 151.9 (C-15 of pyrazoline), 151.2 (C-5 of furan), 146.8 (C-N_3, C-22), 142.0 (C-2 of Furan), 134.2, 130.4, 129.8, 128.2, 124.7, 122.6, 117.6, 110.3 (C-3), 106.3 (C-4 of furan), 58.8 (C-12 of pyrazoline), 54.2 (CH_2, C-25), 40.0 (CH_2 of pyrazoline, C-16); MS (m/z): 440.47 (M-H); Anal.calcd. for $C_{25}H_{17}N_7O_5$ Calculated C: 60.61, H: 3.46, N: 19.79. Found C: 60.55, H: 3.24, N: 19.70.

1-(2-(3-(4-azidophenyl)-5-(4-chlorophenyl)-4,5-dihydro-1H-pyrazol-1-yl)-2-oxoethyl)indoline-2,3-dione (5d)
Yellow solid; yield: 74%; mp: 119-124^{0}C; IR (KBr, cm^{-1}): 1735 (C=O of isatin), 1673 (C=O of isatin), 1624 (C=O of amide), 1558(C=N) and 750 (C-Cl) cm^{-1}; ^{1}HNMR (DMSO-*d*6, 400 MHz, δ ppm): 7.0-7.8 (12H, Ar-H), 5.3-5.5 (dd, 1H, pyrazoine), 4.9-5.1 (s, 2H, CH_2CO), 3.6-3.8 (dd, 1H, pyrazoline), 3.0 (dd, 1H, pyrazoline); ^{13}C NMR (DMSO-d_6, 100MHz, δ ppm): 186.8 (C=O of isatin, C-23), 171.2 (C=O of N-C=O, C-6), 160.8 (C=O of isatin, C-22), 151.2 (C-3 of pyrazoline), 146.8 (C-N_3, C-11), 134.2, 132.4 (C-Cl, C-17), 130.4, 129.8, 128.2, 124.7, 122.6, 114.2, 58.8 (C-5 of pyrazoline), 54.0 (CH_2, C-20), 40.0 (CH_2 of pyrazoline, C-4); MS (m/z): 484.47 (M-H); Anal.calcd. for $C_{23}H_{16}N_6O_4Cl$ Calculated C: 62.72, H: 3.66, N: 19.08. Found C: 61.71, H: 3.50, N: 19.00.

1-(2-(3-(4-azidophenyl)-5-(4-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl)-2-oxoethyl)indoline-2,3-dione (5e)
Yellow solid; yield: 85%; mp 123-126^{0}C; IR (KBr, cm^{-1}): 1728 (C=O of isatin), 1658 (C=O of isatin), 1626 (C=O of amide), 1515&1341 (NO_2 str); ^{1}HNMR (DMSO-d_6, 400 MHz, δ ppm): 7.0-7.8 (12H, Ar-H), 5.2-5.6 (dd, 1H, pyrazoline), 5.0-5.1 (s, 2H, CH_2CO), 3.7-3.9 (dd, 1H, pyrazoline), 3.1 (dd, 1H, pyrazoline); ^{13}C NMR (DMSO-d_6, 100MHz, δ ppm): 184.9 (C=O of isatin, C-23), 171.4 (C=O of N-C=O, C-6), 161.0 (C=O of isatin, C-22), 151.2 (C-3 of pyrazoline), 146.9 (C-N_3, C-11), 146.5 (C-NO_2, C-17), 134.5, 130.4, 129.4, 128.5,124.3, 122.7, 117.8, 58.6 (C-5 of pyrazoline), 54.0 (CH_2, C-20), 40.0 (CH_2 of pyrazoline, C-4); MS (m/z): 495.43(M-H); Anal.calcd. for $C_{25}H_{17}N_7O_3$ calculated C: 54.57, H: 21.43, N: 21.16. Found C: 54.55, H: 21.40, N: 21.08.

PHARMACOLOGICAL SCREENING
EVALUATION OF IN VITRO ANTI-CANCER ACTIVITY
All the synthesized compounds were subjected to MTT assay against three cancer cell lines (NCI-H23, MCF-7 and HCT-116) at 100, 50, 25 & 12.5 µM concentrations. Nevertheless, compound **5a** possessed considerable activity and was tested on to further evaluation of concentrations 6.25, 3.125 and 1.562 µM. The IC_{50} values of the compounds were recorded and shown in **table-1**. Among the five compounds screened, compound **5a** showed highest activity against NCI-H23 & MCF-7 cell lines with IC_{50} values at 1.06 & 3.92 µM respectively. Compounds **5b, 5c** & **5d** have shown activity against all the three cell lines with IC_{50} values ranging between 20 – 55 µM, while **5e** exhibited moderate activity with IC_{50} values above 100 µM.

Table-1: ***In vitro*** **cytotoxic activity of synthesized compounds against three cell lines**

Compound	Ar	$IC_{50}(\mu M)^a$		
		NCI-H23	MCF-7	HCT-116
Doxorubicin	-	2.24 ± 0.02	3.82 ± 0.06	3.91 ± 0.04
5a	4-OCH3-C6H4	1.06± 0.12	3.92±0.16	19.87±0.41
5b	4-F-C6H4	20.53±0.23	23.74±0.69	24.19±0.58
5c	Furyl	29.15±0.49	35.64±0.34	48.12±0.67
5d	4-Cl-C6H4	21.13±0.52	32.97±0.82	54.35±0.81
5e	4-NO_2-C6H4	>100	>100	>100

[a] *Mean value ± standard deviation from 3 independent experiments.*

EVALUATION OF IN VIVO ANTITUMOR ACTIVITY AGAINST EAC

Based on promising results obtained in early *in vitro* assay, all the compounds were screened for *in vivo* anticancer activity against EAC bearing mice. The reduction in viable tumor cell count, MST and % ILS are important measures that were used in this *in vivo* testing. 5-Flurouracil (20 mg/kg body weight) was used as standard drug. The results were shown in Table 2.

Table 2: ***In vivo*** **anti proliferative activity of compounds 5a, 5b, 5c, 5d and 5e**

Group	MST (days) ± SE[a]	ILS (%)	Tumor volume (mL)	Viable cells in ascetic fluid (%)
I	-	-	-	-
II	17.9 ± 0.38	-	3.2 ± 0.2	92.8 ± 3.2
III	35.7 ± 0.51*	96.57	0.8 ± 0.02	16.2 ± 1.7
IV	31.5 ± 0.48*	73.47	1.8 ± 0.02	35.7 ± 1.4
V	28.3 ± 1.46*	58.86	2.0 ± 0.03	39.2 ± 1.2
VI	23.6 ± 0.93*	34.95	2.6 ± 0.05	50.1 ± 1.2
VII	26.8 ± 1.32*	56.13	2.1 ± 0.05	42.7 ± 1.5
VIII	16.6 ± 0.41*	28.17	2.9 ± 0.07	48.6 ± 1.3

[a] *Standard error; Significant values are compared to the control group (n=5),* *$P<0.001$*

EFFECT ON SURVIVAL TIME

All *in vivo* tested compounds showed significant increase in MST and % ILS as compared to EAC control **(Table 2)**. Compound **5a** exhibited highest antitumor activity and showed 31.5 ± 0.48 days of MST with 73.47 ILS as compared to rest of the compounds. Compound **5b** and **5d** showed 28.3 ± 1.46 and 26.8 ± 1.32 days of MST with 58.86 and 56.13 % ILS respectively. Compound **5c** showed moderate activity with 23.6 ± 0.93 days of MST and 34.95 % ILS whereas compound **5e** exhibited less activity with 16.6 ± 0.41 days of activity and 28.17 % ILS as compared to all other compounds.

TUMOR VOLUME AND VIABLE CELLS

Antitumor activity of synthesized compounds on EAC bearing mice *in vivo* was further studied in terms of tumor volume and number of viable cells. Almost all the compounds were exhibited significant decrease in tumor volume and cell number as compared to EAC control. Compound **5a**, which showed highest % ILS, demonstrated maximum reduction of viable cells (35.7 ± 1.4) and tumor volume (1.8 ± 0.02) as rest of the compounds.

EVALUATION OF ANTI-BACTERIAL ACTIVITY

All the synthesized compounds were evaluated for anti-bacterial activity against four organisms at concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml using cup-plate method. The MIC values of the compounds were recorded and shown in the table-2. As per the data, compounds **5b**& **5d** showed highest activity against Gram-negative bacteria i.e. *Pseudomonas aeruginosa* (NCTC 10662) *&* *Escherichia coli* (NCTC 10418) with MIC values in the range of 6.25-25 µg/ml. However, compounds **5a**& **5e** exhibited moderate activity with MIC values between 50-100 µg/ml while **5c** showed MIC values above 100 µg/ml against all the four bacteria.

Table 3: MIC values (µg/mL) of synthesized compounds against Gram-positive and Gram-negative bacterial strains

Compound	***Staphylococcus aureus*** **(NCTC 6571)**	***Bacillus subtilis*** **(NCTC 10400)**	***Pseudomonas aeruginosa*** **(NCTC 10662)**	***Escherichia coli*** **(NCTC 10418)**
5a	100	50	50	100
5b	50	25	6.25	12.5
5c	>100	>100	>100	>100
5d	>100	25	12.5	25
5e	100	50	50	100
Ciprofloxacin	<5	<1	<5	<1

MIC-Minimum Inhibitory Concentration

CONCLUSION

In the present study, new isatin linked pyrazoline conjugates have been synthesized and characterized by spectral data. All the five compounds were evaluated for anti-cancer and anti-bacterial activities. Among the five compounds, compound **5a** possessing para methoxy substituent on phenyl ring has emerged as a highly potent compound in *in vitro* and *in vivo* anti-cancer studies. Moreover, a good correlation among the results was obtained in *in vitro* and *in vivo* studies. Compound **5e** with an electron withdrawing NO_2 group showed mild anticancer activity in both the studies. In anti-bacterial screening, compounds **5b & 5d** exhibited significant activity against Gram- negative bacteria with MIC values between 6.25-25.0 µg/ml. Hence, further investigation of such isatin derivatives will be quite useful with the hope of arriving at lead compounds possessing selective anti-cancer & anti-bacterial properties.

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